Synthesis of Polymerized Human Serum Albumin

Undergraduate Honors Thesis

Presented in Partial Fulfillment of the Requirements for

Graduation with Honors and Distinction

at The Ohio State University

By

Qi Wang

William G. Lowrie Department of Chemical and Biomolecular Engineering

The Ohio State University

Spring, 2011

Oral Defense Committee Members:

Andre F. Palmer, Advisor

David Wood

Abstract

In the event where blood for transfusion is unavailable, plasma expanders are often used to treat patients with significant blood loss. The use of many plasma expanders is often limited by their undesirable side effects. Human serum albumin (HSA) has been an attractive plasma expander. However, despite it being an important component in the blood, it can increase the risk of mortality when administered to patients with increased vascular permeability, such as in victims of severe burn injury, septic shock and endothelial dysfunction. This is caused by HSA extravasation, where HSA in the blood leaks to the surrounding tissues. This harmful side effect of HSA extravasation can be greatly reduced if the molecular size of HSA is increased. In this study, HSA was non-specifically cross-linked with glutaraldehyde. The polymerized HSA (PolyHSA) made was stabilized by a quenching reaction with $NaBH_4$, and purified with diafiltration. The molecular weight and viscosity of the polymerized HSA increases with increasing cross-link density, and the colloid osmotic pressure (COP) decreases with increasing cross-link density. Circular dichroism shows that the secondary structure of HSA is conserved after polymerization. Altogether, these results show that glutaraldehyde can effectively cross-link HSA, significantly increase its molecular size, yielding a series of novel potential plasma expanders with high molecular weight and viscosity that prevent the harmful effects of HSA extravasation.

Acknowledgments

I would like to thank my adviser, Professor Palmer, for all the support and advice he has given me over the years. Without his wisdom and help, this project would not have been possible.

I would also like to thank Jacob Elmer for his guidance and insight which helped me tremendously in understanding the sciences behind this project.

A special thanks to Ning Zhang, who provided help with the data acquisition for molecular weight distributions using light scattering.

This research is supported in part by an undergraduate honors research scholarship awarded by The Ohio State University College of Engineering.

Publications

Jacob Elmer, Pedro Cabrales, Qi Wang, Ning Zhang, and Andre F. Palmer.

'Synthesis and Biophysical Properties of Polymerized Human Serum Albumin."

Biotechnology Progress. 2011; 27 (1): 290-296.

Fields of Study

Major Field: Chemical and Biomolecular Engineering

Table of Contents

List of Figures

List of Tables

I. Introduction

1. Background on Plasma Expanders

When blood is lost, the immediate need is to stop the blood loss, and replace the lost volume. When blood for transfusion is unavailable, plasma expanders (PEs) are commonly used to treat patients with significant blood loss. Unlike blood transfusions, which increase blood's oxygen carrying capacity by adding more red blood cells (RBCs), PEs increase the oxygen delivery of the remaining RBCs by restoring the circulatory volume and maintaining adequate blood flow. Normal human blood has a significant excess oxygen transport capability. Patients in their resting state can safely tolerate a very low hemoglobin concentration, about 50 g/L, compared to 150 g/L for the normal blood hemoglobin concentration[1,2]. The decrease in red blood cell concentration increases the shear rate, which stimulus the endothelium to signal the dilation of blood vessels, maintaining systemic oxygen delivery[3].

Effectiveness of PE to stay within the intravascular space depends on its colloid osmotic pressure (COP) and molecular size. Molecules smaller than 50 kDa in size are rapidly excreted out of the body via kidneys. These molecules are also susceptible for rapid diffusion to the surrounding tissue space[4]. Intravascular COP must be kept either above the capillary hydrostatic pressure or at least greater than 10 mm Hg to maintain sufficient blood flow[5]. Depending on COP and size of PE molecule, this typically requires more infused volume of PE than the volume that is actually needed. The amount of PE needs to be infused can be reduced by using PEs with larger molecular size or higher COP. The increase in colloid osmotic pressure causes fluids to transfer from the

tissue space into the circulatory system, increasing the circulatory volume. This increase in circulating blood volume stabilizes the patient by restoring microvascular blood flow and capillary perfusion[6].

2. Types of Plasma Expanders

There are several types of PEs that are currently in use. For example, crystalloid-based saline (0.9%), colloids like gelatin, dextran, hydroxyethyl starch, and human serum albumin[7]. However, the use of them many cause undesirable side effects.

Crystalloids and colloids based solutions are the established procedure for primary volume replacement in patients with hemorrhagic shock[8]. Examples of crystalloid based solutions are dextrose and crystalloid-based saline (0.9%). Crystalloid based solutions are inexpensive, but their effectiveness lack endurance, and need to be continually supplied[8]. These solutions acts as free water after intravenous administration, and is quickly metabolized. They rapidly equilibrate between the intracellular and extracellular space. For every 100 mL of an intravenous infusion of saline solution, about three-quarters will pass into the interstitial space within the first hour, and only one-quarter remains in the intravascular space. Only 5-7 mL remains in the intravascular space for every 100 mL of dextrose solutions infused[5].

Colloid derived plasma expanders can be based from either natural (human serum albumin), and synthetic (dextrans, hydroxyethyl starches) macromolecules. Since colloids are larger in size, their retention rate within the intravascular space is better than crystalloids. However, colloids may still leak out from capillaries to the surrounding tissue space, increasing the interstitial colloid osmotic pressure, which causes a negative

effect of increasing the fluid flux out from blood vessels[5]. Colloids like gelatins have poor volume expansion and may cause allergic reactions[9]. Other examples of colloid based PEs are Dextran and hydroxyethyl starch.

Dextran is a complex, branched polysaccharide composed of chains of varying length. Hydroxyethyl starch (HES) is a type of synthetic colloid derived from amylopectin. These types of PEs can remain effective for up to 24 hours[5]. Dextrans and hydroxyethyl starches have been shown to be able to restore circulatory volume and microvascular perfusion[10,11]. However, despite their benefits, the use of these types of PEs is limited by their negative impact on red blood cells aggregation, and acute renal failure[12,13]. Another disadvantage of hydroxyethyl starch solutions is that they contain molecules with a wide range of molecular weights, and the smaller molecules will be rapidly excreted from the body, decreasing its effectiveness[5].

Human serum albumin (HSA) is a type of naturally based colloid derived from donated blood, and the use of it as a PE has many advantages. It is naturally produced in the liver, makes up ~50% of the total plasma protein[14], provides ~75% of the plasma's COP[15], and has been shown to be generally safe[16]. When administered, its duration of effectiveness within the intravascular space can be up to a day, and it persists within the body for about 20 days[5]. However, HSA has also been shown to increase the risk of morality in patients with severe burn injury and sepsis. This is likely due to HSA extravastion in patents with increased vascular permeability[17,18]. Extravasation of HSA can cause edema, reduce plasma COP, and expose tissues to toxins bound by HSA.

3. Objectives and Experimental Summary

The objectives of this project are to produce a series of novel plasma expanders from HSA and glutaraldehyde, having high molecular weight, high viscosity, and low COP. Higher molecular weight PolyHSA can limit extravasation, a harmful effect of monomeric HSA PE in patents with increased vascular permeability[19]. Higher viscosity can induce vasodilation and increased microvascular perfusion[20,21]. Lower COP can control the volume expansion of PolyHSA to be within a safe level.

In this study, HSA was polymerized with glutaraldehyde at glutaraldehyde to HSA molar ratios of 24:1, 60:1 and 94:1, and stabilized by a quenching reaction with $NaBH_4$. The PolyHSA made was then purified with tangential flow filtration. Weight averaged molecular weight distribution, secondary structure, viscosity, and COP was measured to assess its potential as a novel plasma expander.

II. Background

Payne et al. polymerized bovine serum albumin (BSA) with various concentrations of glutaraldehyde[22]. They showed that it is possible to increase the molecular size of BSA by reacting it with glutaraldehyde. However, is that study, a reducing agent, such as $NaBH_4$, was not used to stabilize the polymerized BSA, making it susceptible to degradation back to free BSA. In a study done by Cabrales et al., HSA was covalently conjugated with polyethylene glycol (PEG), and was shown to exhibit a longer circulatory half-life and increased blood volume expansion function[23].

Glutaraldehyde had previously been used to crosslink hemoglobin (Hb) to yield a large Hb-based oxygen carrier. There are two commercial glutaraldehyde cross-linked Hbs available[24]. HBOC-201 is a glutaraldehyd polymerized bovine Hb developed by Biopure Corp.Cambridge, MA, with an average molecular weight (MW) of 250 kDa. PolyHeme, developed by Northfield Labs, Evanston, IL, is a glutaraldehyde polymerized human Hb with an average MW of 150 kDa. Recently, HBOC-201 had shown promising results in phase III clinical trials[25].

Studies had shown that there are many benefits associated with the use of high viscosity PEs[26,27]. The use of high-viscosity plasma expanders better maintain capillary perfusion and microvascular flow in extreme cases of hemorrhagic shock[8]. High viscosity plasma expanders also maintain the functional capillary density (FCD), making the use of them in patients with extreme anemia more desirable[8]. FCD is the number of functional capillaries for passage of red blood cells per unit surface of tissue, and it has been found by Kerger et al. that this parameter is important in defining tissue survival[28].

PolyHSA's advantage of having higher viscosity and molecular weight, together with HSA's benefit of being naturally prevalent in the blood, shows its potential of being a series of novel plasma expanders that prevents the harmful side effects of other types of PEs.

III. Materials and Methods

1. Glutaraldehyde Cross-linking Method

Glutaraldehyde produces protein polymers with high molecular weight and viscosity by reacting with the lysine, histidine, tyrosine, arginine, and primary amine groups of the protein. It forms both intra and intermolecular cross-links[29]. HSA has several of those residues, especially lysine, which is solvent exposed and available for polymerization with glutaraldehyde, shown in Figure 1[30].

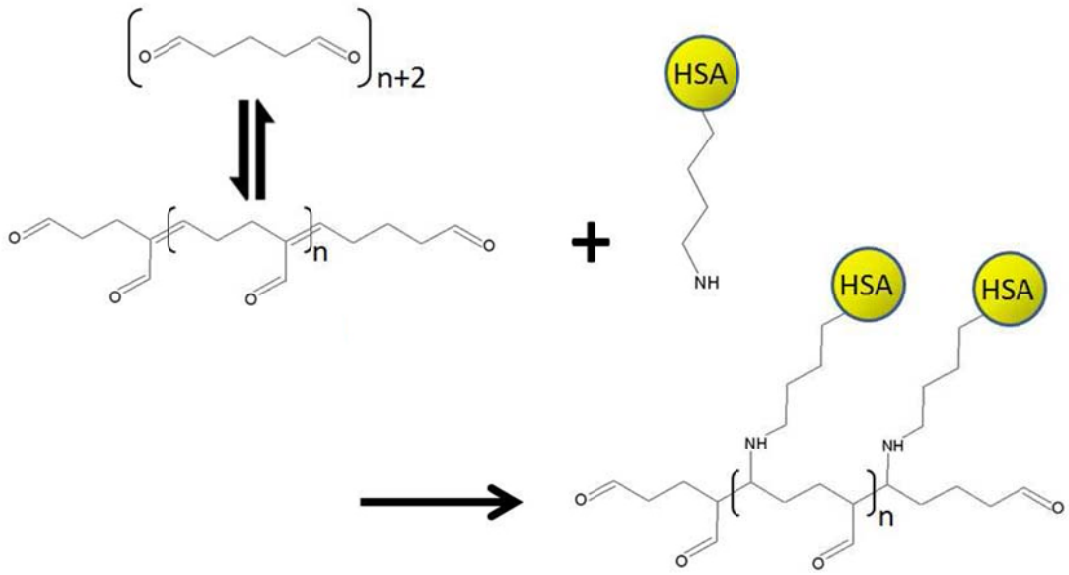

Figure 1: Reaction scheme of glutaraldehyde with itself and intermolecular reaction with lysine residues on the surface of HSA.

2. Materials

AlbuminarVR -25 (HSA) was purchased from ABO Pharmaceuticals (San Diego, CA) at a concentration of 250 mg/mL. Glutaraldehyde (70 wt%) was purchased from Sigma Aldrich (Atlanta, GA). Other chemicals including $NaBH_4$, NaCl, NaOH, HCl, KCN, sodium lactate, N-acetyl-L-cysteine, NaH_2PO_4, and Na_2HPO_4 were purchased from Fisher Scientific (Pittsburgh, PA). KrosFlow Research II tangential flow filtration (TFF) system and two 500 kDa hollow fiber (HF) filter modules were obtained from Spectrum Laboratories (Rancho Dominguez, CA). Those HF filters were made from polysulfone with a total membrane surface area of 615 cm^2.

3. PolyHSA Synthesis

For synthesis of PolyHSA, 100 mL of HSA (250 mg/mL) was diluted to 25 mg/mL with phosphate buffered saline (PBS) (1.42 gm Na_2HPO_4, 8.18 gm NaCl, and 0.75 mg KCl per liter, pH = 7.4) up to a final volume of 1 L. Glutaraldehyde (70 wt%) was added to HSA solutions at the following molar ratios of glutaraldehyde to HSA: 24:1 (HSA_{24}), 60:1 (HSA_{60}), and 94:1 (HSA_{94}). The polymerization reaction was incubated at 37^oC for 3 hours, then quenched with 25 mL of 1 M sodium borohydride and incubated for an additional 30 minutes.

4. PolyHSA Purification

For purification of PolyHSA, diafiltration was used to eliminate the presence of free glutaraldehyde and other impurities in the final product, PolyHSA solutions were diafiltrated with 2 L of modified lactated Ringer's buffer (115 mM NaCl, 4 mM KCl, 1.4

mM $CaCl_2$, 13 mM NaOH, 27 mM sodium lactate, and 2 g/L N-acetyl-L-cysteine) with two 500 kDa hollow fiber filters for 4 times, and concentrated to a final volume of 200 mL. The process flow of the filtration process is shown in Figure 2. At each filtration cycle, filtrate was collected, and the retentate was recycled to maximize the yield of PolyHSA.

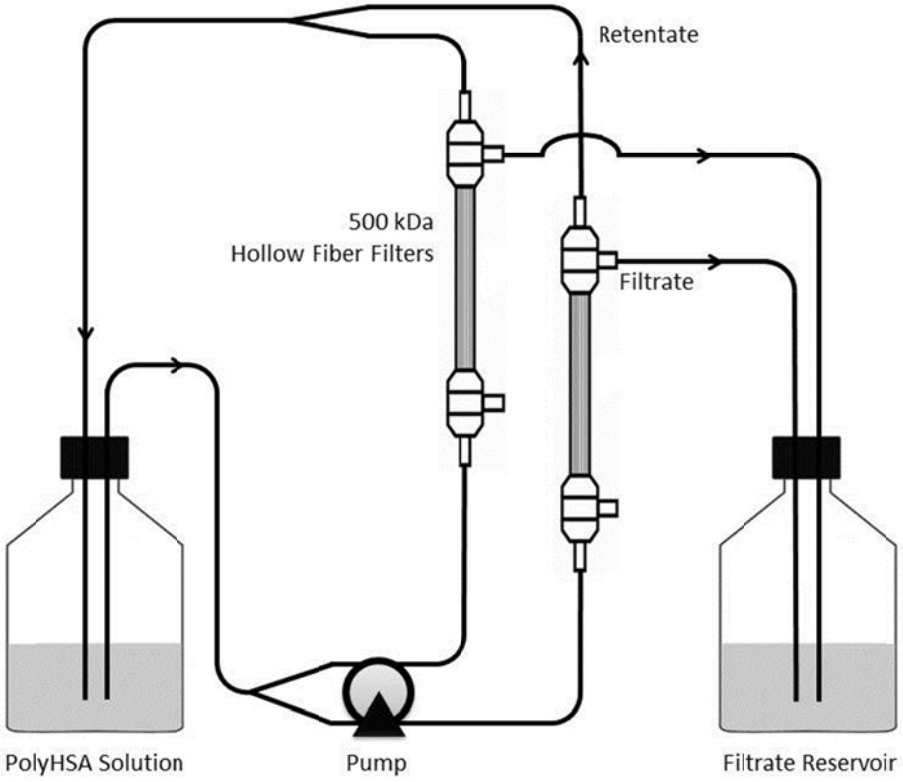

Figure 2: Process Flow of the Filtration Step.

PolyHSA solutions synthesized were then sterile filtered through 0.2 μm filters (the 94:1 Poly-HSA sample could not be sterile filtered because of its high MW and viscosity), and stored at -80^{o}C until needed. Polymerizations at each HSA to glutaraldehyde molar ratios were repeated three times to ensure data consistency.

5. PolyHSA Characterization

The increase of molecular weight (MW) of PolyHSA was shown by SDS-PAGE Analysis. The absolute MW distribution of PolyHSA solutions was measured using light scattering photometer. Circular Dichroism was used to compare the secondary structure of PolyHSA with HSA.

Viscosity measurements of HSA/PolyHSA solutions were conducted in a cone/plate viscometer DV-II plus, with a cone spindle CPE-40 (Brookfield Engineering Laboratories, Middleboro, MA) at a shear rate of 160 s^{-1}, and the COP of polyHSA was measured with a Wescor 4420 Colloid Osmometer (Wescor, Logan, UT).

For Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) analysis, protein (25 μg) from each HSA/PolyHSA solution was mixed with Lammeli buffer (1:1 ratio), incubated at 95^{o}C for 5 min, and ran on a polyacrylamide gel (12% resolving gel and 4% stacking gel) at 110 V for 1.5 hrs. The gel was then stained with Coomassie blue for ~12 hrs, destained with destaining solution (20% ethanol and 10% acetic acid), and visualized on a Gel Doc XR imaging system (BioRad Hercules,CA).

For measuring the absolute molecular weight distribution of HSA/PolyHSA solutions, light scattering instruments consisting of a size exclusion column (Ultrahydrogel linear column, 10 μm, 7.8x300 mm; Waters, Milford, MA) driven by a 1200 HPLC pump (Agilent, Santa Clara, CA), controlled by Eclipse 2 software (Wyatt Technology, Santa Barbara, CA) connected in series to a DAWN Heleos (Wyatt Technology) light scattering photometer and an OptiLab Rex (Wyatt Technology) differential refractive index detector was used. The mobile phase consisted of 20 mM

phosphate buffer (pH 8.0), 100 ppm NaN3, and 0.2 M NaCl (Fisher Scientific) in HPLC grade water that was filtered through a 0.2 μm membrane filter. HSA and PolyHSA solutions were diluted to 1 mg/mL with the mobile phase, and 100 μL of the sample was injected into the column via a 1200 Autosampler (Agilent). All data were collected and analyzed using Astra 5.3 (Wyatt Technology) software.

Circular dichroism (CD) spectra were obtained on an AVIV Circular Dichroism Spectrophotometer Model 202 (Aviv Biomedical Lakewood, NJ). HSA and PolyHSA solutions were diluted with PBS to ~80 μg/mL. The cell temperature was maintained at 25^{o}C and each spectrum was averaged over three consecutive measurements taken at 1 nm intervals from 200 to 250 nm.

IV. Results

SDS-PAGE showing MW distribution of HSA/PolyHSA solutions is shown in Figure 3. The absolute MW distribution of HSA/PolyHSA solutions measured using size exclusion chromatography coupled with static light scattering is shown in Figure 4, and the weight averaged MW of HSA and PolyHSA solutions based on triplicate reactions is reported in Table 1. Circular Dichroism (CD) used to compare the secondary structure of PolyHSA solutions with HSA is shown in Figure 5. The viscosity of HSA/PolyHSA solutions, and the COP of PolyHSA solutions is shown in Table 2.

1. SDS-PAGE Analysis

Glutaraldehyde polymerized HSA produced a wide distribution of large MW molecules. Monomeric HSA used as control, shows a strong band at around 67 kDa, the literature reported HSA monomer MW, and several faint bands at lower MWs (degraded proteins). Each of the PolyHSA solutions displays intense broad bands above 100 kDa. It is evident that the MW of PolyHSA increases with increasing cross-link density. There are some polymerized HSA present in each of the PolyHSA solutions. However, the amount of HSA monomer decreases as the glutaraldehyde:HSA molar ratio (cross-link density) increases.

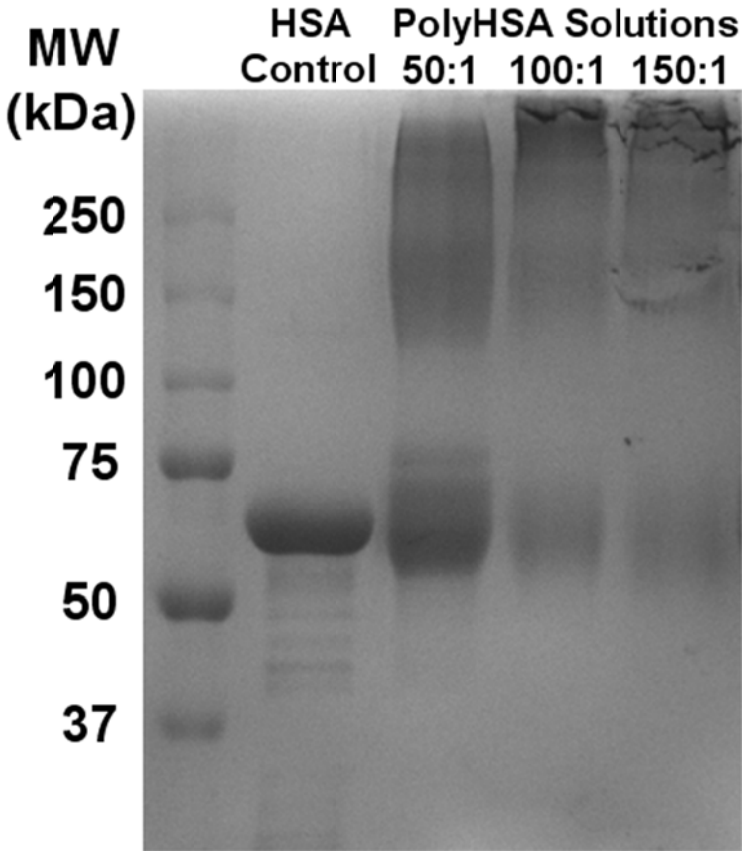

Figure 3: SDS-PAGE analysis of HSA and PolyHSA solutions.

2. Light Scattering

The MW distributions measured with light scattering agrees with the SDS-PAGE) results. MW of PolyHSA increases with increasing cross-link density. The observed weight averaged MW of the control monomeric HAS, at 70 kDa, is very close to the literature rqeported MW (67 kDa). Increases in weight averaged MW of the PolyHSA changes non-linearly with increasing cross-link density, from a modest change seen with HSA_{24} (243 kDa) to the large increase observed in HSA_{94} (11800 KDa).

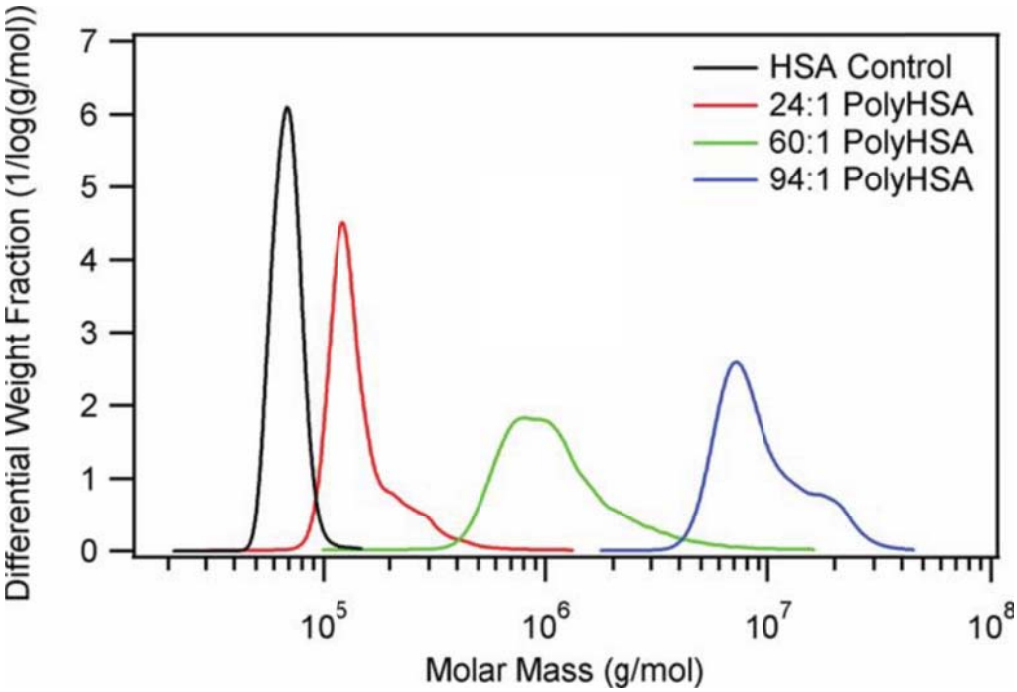

Figure 4: Absolute MW distribution of HSA and PolyHSA solutions.

Solution	Weight Averaged MW (kDa)
HSA Control	90 ± 13.7
24:1 PolyHSA	243 ± 60.3
60:1 PolyHSA	1,997 ± 102
94:1 PolyHSA	11,839 ± 2,669

Table 1: Weight averaged MW of HSA and PolyHSA solutions based on 3 trials.

3. Circular Dichroism

The CD spectra of PolyHSA samples are highly similar to HSA. It is evident that the secondary structure of HSA is unaffected by the extent of polymerization reaction.

For monomeric HSA, it exhibits a strong minimum around 212 and 224 nm, indicating the presence of alpha helices, which account for 60% of the secondary structure of HSA.

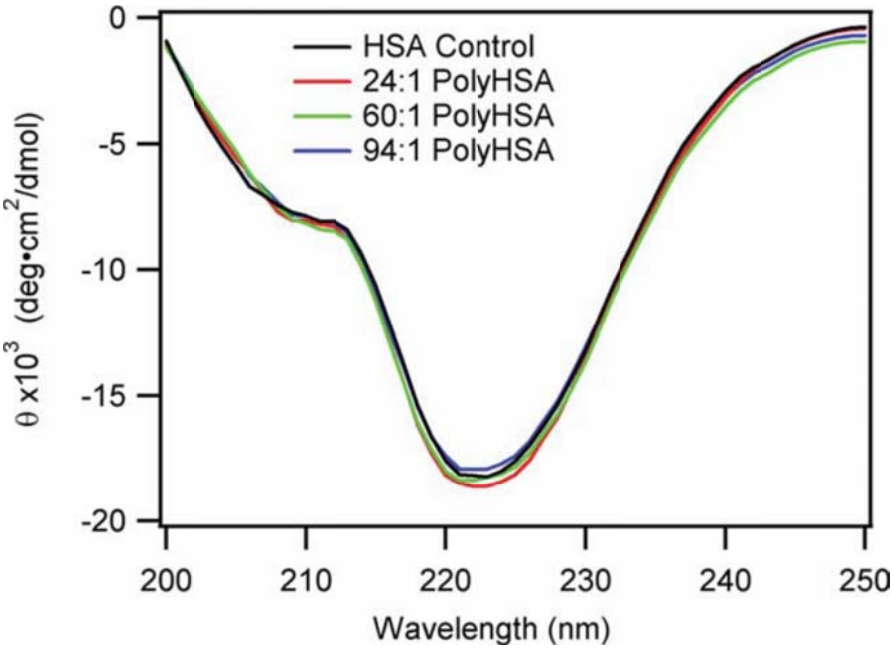

Figure 5: CD spectra of HSA and PolyHSA solutions at 37°C.

4. Viscosity and COP

The cross-link density has a significant effect on the viscosity and COP of the final PolyHSA solution. Viscosity of PolyHSA solutions increase with increasing cross-link density, while in contrast, the COP of the solutions decreases as the cross-link density increases. The viscosity of each PolyHSA solution is higher than that of monomeric HSA (1.4 cP), ranging from 1.6 cP (HSA24) to 15.2 cP (HSA94). The increase in viscosity becomes more dramatic as cross-link density increases.

A 50% decrease in COP is observed with HSA_{24} compared to the control HSA, whereas HSA_{60} and HSA_{94} have an almost insignificant COP (4 and 1 mm Hg, respectively).

Sample	Viscosity (cp)	COP (mm Hg)
HSA Control	1.4	42
24:1 PolyHSA	1.6	22
60:1 PolyHSA	11.2	4
94:1 PolyHSA	15.2	1

Table 2: Viscosity and COP of HSA and PolyHSA solutions at 10 g/dL.

V. Discussion

1. Effects of Polymerization on MW and Secondary Structure

SDS-PAGE analysis and light scattering results show that glutaraldehyde can be effectively used to polymerize HSA at cross-link densities of 24:1, 60:1, and 94:,1 to yield a solution of high MW polymers. As expected, increasing the cross-link density increases the weight averaged MW of the PolyHSA solution. The CD spectra show that the reaction of glutaraldehyde with HSA does not affect the secondary structure of HSA, so the protein does not unfold, preserving its functions, such as toxin binding.

2. Effects of Polymerization on Viscosity and Colloid Osmotic Pressure

The viscosities of PolyHSA solutions are higher than that of HSA at the same total protein concentration at 10 g/dL, Viscosity also increases as cross-link density increases. This is likely due to the large increase in the weight averaged MW of the PolyHSA solutions, which increases the frequency of molecular interactions between neighboring PolyHSA molecules, causing their viscosities to increase.

COP of the PolyHSA solutions decreases with increasing cross-link density and is significantly lower than that of HSA. This significant reduction in COP is probably due to the fact that COP is primarily determined by the presence of unpolymerized HSA and small HSA polymers in solution. As cross-link density increases, PolyHSA molecules get larger, and the number of unreacted HSA decreases, which in turn reduces the COP.

3. Effects of PE Viscosity

It has been believed that lowering blood viscosity leads to an overall health benefit by decreasing vascular resistance and heart workload[31]. However, on the contrary, PEs with high viscosity increases the shear stress of the blood vessel walls, which induces endothelial cells to produce nitric oxide (NO) that in turn dilates blood vessels[32,33]. NO is a critical regulator of vascular homeostasis and controls vascular relaxation[34,35]. This dilation in blood vessel makes oxygen transport to the sounding tissues easier and increases microvascular perfusion. High viscosity PEs can be used to preserve normal blood viscosity in cases of acute drop in blood volume[36].

In an animal study done by Tsai et al., it was shown that the use of a high viscosity, low COP PE allows a better maintenance of stable hemodynamic conditions in cases of extreme anima[26]. In that experiment, a high viscosity PE was used to increase the blood viscosity without simultaneously increasing the colloid oncotic pressure (COP) to viscosities beyond the normal blood viscosity. It was discovered that the high blood viscosity group had a significantly higher functional capillary density and an increase in blood flow.

Studies have shown that the viscosity of a PE can be increased with increasing concentration[26]. However, there is a physiological limitation to this self-limiting approach. Increasing the PE concentration also increases the COP[37]. Since higher COP increases the amount of extravascular fluid being pulled into the intravascular space, this not only makes it harder to control the amount of PE needs to be infused, it also dilutes the blood, reducing the blood viscosity to a point lower than the desired value.

VI. Recommendations

Despite the many benefits associated with the use of high viscosity PEs, especially in the treatment of extreme cases of hemorrhagic shock and anemia. High viscosity PEs also have negative effects. For example, increasing the blood viscosity will also rapidly increase vascular resistance, causing the heart workload to increase[31]. Therefore the use of high viscosity PolyHSA PEs should be leveraged with their negative effects.

Because of PolyHSA's high viscosity and low COP properties, it is recommended that PolyHSA to be formulated with HSA or other type of plasma expanders in cases where volume expansion is required. It can also be used to increase the viscosity of the blood while keeping the COP low, making it an ideal PE for the treatment of extreme anemia and extreme cases of hemorrhagic shock. Animal studies with glutaraldehyde cross-linked HSA could be used to determine its effects on functional capillary density, microvascular perfusion, and general recovery after hemorrhagic shock.

VI. Conclusions

The results of this study show that glutaraldehyde can be used to produce a high MW HSA polymer with conserved secondary structure, high viscosity, and low COP. The large MW of PolyHSA should limit extravasation and its high viscosity should induce vasodilation and increased microvascular perfusion. The low COP may limit volume expansion of PolyHSA beyond the infused volume, but the large size of PolyHSA should ensure the effectiveness of the infused volume within the intravascular space.

References

1. Welch HG, Meehan W, Goodnough LT. **Prudent strategies for elective red blood cell transfusion.**

   Ann Int Med. 1992;116:393-402.

2. Beutler E, Waalen J. **The definition of anemia: what is the lower limit of normal of the blood hemoglobin concentration?**

   Blood. 2006;107:1747-1750.

3. Doss DN, Estafanous FG, Ferrario CM, Brum JM, Murray PA. **Mechanism of systemic vasodilation during normovolemic hemodilution**.

   Anesth Analg. 1995;81:30-34.

4. Webb AR, Barclay SA, Bennett ED. **In vitro colloid osmotic pressure of commonly used plasma expanders and substitutes: a study of the diffusibility of colloid molecules.**

   Intensive Care Med. 1989;15:116-120.

5. Huskisson L. **Intravenous volume replacement: which fluid and why?**

   Archives of Disease in Childhood. 1992;67:649-653.

6. Cabrales P, Nacharaju P, Manjula BN, Tsai AG, Acharya SA, Intaglietta M. **Early difference in tissue pH and microvascular hemodynamics in hemorrhagic shock resuscitation using polyethylene glycol-albumin- and hydroxyethyl starch-based plasma expanders**.

   Shock. 2005;24:66-73.

7. Kreimer U, Messmer K. **Perioperative hemodilution**.

Transfus Apher Sci. 2002;27:59-72.

8. Cabrales P, Tsai AG, Intaglietta M. **Hyperosmotic-hyperoncotic vs. hyperosmotic-hyperviscous small volume resuscitation in hemorrhagic shock**.

   Shock. 2004;22:431-437.

9. Ljungstrom, K. **Colloid safety: fact and fiction**.

   Baillieres Clin Anaes. 1997;11:163-177.

10. Cabrales P, Tsai AG. **Plasma viscosity regulates systemic and microvascular perfusion during acute extreme anemic conditions**.

    Am J Physiol Heart Circ Physiol. 2006;291:H2445-2452.

11. Vollmar B, Lang G, Menger MD, Messmer K. **Hypertonic hydroxyethyl starch restores hepatic microvascular perfusion in hemorrhagic shock**.

    Am J Physiol Heart Circ Physiol. 1994;266:H1927-H1934.

12. McCahon R, Hardman J. **Pharmacology of plasma expanders**.

    Anaesth Intens Care Med. 2007;8:79-81.

13. Chien S, Jan K-M. **Red cell aggregation by macromolecules: roles of surface adsorption and electrostatic repulsion**.

    J Supramol Struct. 1973;12:385-409.

14. Quinlan GJ, Martin GS, Evans TW. **Albumin: biochemical properties and therapeutic potential**.

    Hepatology. 2005;41:1211-1219.

15. Kragh-Hansen U, Chuang VT, Otagiri M. **Practical aspects of the ligand-binding and enzymatic properties of human serum albumin**.

Biol Pharm Bull. 2002;25:695-704.

16. Finfer S, Bellomo R, Boyce N, French J, Myburgh J, Norton R, SAFE Study Investigators. **A comparison of albumin and saline for fluid resuscitation in the intensive care unit**.

N Engl J Med. 2004;22:2247-2256.

17. Fleck A, Raines G, Hawker F, Trotter J, Wallace P, Ledingham I, Calman KC. **Increased vascular permeability: a major cause of hypoalbuminaemia in disease and injury**.

Lancet. 1985;8432:781-784.

18. Haupt MT, Kaufman BS, Carlson RW. **Fluid resuscitation in patients with increased vascular permeability**.

Crit Care Clin. 1992;8:341-353.

19. Matheson B, Kwansa HE, Bucci E, Rebel A, Koehler RC. **Vascular response to infusions of a nonextravasating hemoglobin polymer.**

J Appl Physiol. 2002;93:1479-1486.

20. Cabrales P, Intaglietta M, Tsai AG. **Increase plasma viscosity sustains microcirculation after resuscitation from hemorrhagic shock and continuous bleeding**.

Shock. 2005;23:549–555.

21. Tsai AG, Friesenecker B, McCarthy M, Sakai H, Intaglietta M**. Plasma viscosity regulates capillary perfusion during extreme hemodilution in hamster skinfold model**.

Am J Physiol. 1998;275:H2170-2180.

22. Payne J. **Polymerization of Proteins with Glutaraldehyde**.

Biochem J. 1973;135:867-873.

23. Cabrales P, Tsai AG, Ananda K, Acharya SA, Intaglietta M. **Volume resuscitation from hemorrhagic shock with Albumin and Hexa Pegylated Human Serum Albumin**.

Resuscitation. 2008;79:139-146.

24. Zhang N, Palmer AF. **Polymerization of Human Hemoglobin Using the Crosslinker 1,11 Bis(maleimido)triethylene Glycol for Use as an Oxygen Carrier.**

Biotechnol Prog. 2010;26:1481-1485.

25. Jahr JS, Mackenzie C, Pearce LB, Pitman A, Greenburg AG. **HBOC-201 as an alternative to blood transfusion: efficacy and safety evaluation in a multicenter phase III trial in elective orthopedic surgery.**

J Trauma. 2008;64:1484–1497.

26. Cabrales P, Tsai AG, Intaglietta M. **Alginate plasma expander maintains perfusion and plasma viscosity during extreme hemodilution**.

Am J Physiol Heart Circ Physiol. 2005;288:H1708-1716.

27. Cabrales P, Tsai AG, Intaglietta M. **Microvascular pressure and functional capillary density in extreme hemodilution with low- and high-viscosity dextran and a low-viscosity Hb-based O2 carrier**.

Am J Physiol Heart Circ Physiol. 2004;287:H363-373.

28. Kerger H, Waschke KF, Ackern KV, Tsai AG, Intaglietta M. **Systemic and microcirculatory effects of autologous whole blood resuscitation in severe hemorrhagic shock.**

Am J Physiol. 1999;276:2035–2043.

29. Habeeb AJ, Hiramoto R. **Reaction of proteins with glutaraldehyde**.

Arch Biochem Biophys. 1968;126:16-26.

30. Sugio S, Kashima A, Mochizuki S, Noda M, Kobayashi K. **Crystal structure of human serum albumin at 2.5 A resolution**.

Protein Eng. 1999;12:439-446.

31. Lowe G, Rumley A, Norrie J, Ford I, Shepherd J, Cobbe S, Macfarlane P. **Blood Rheology, Cardiovascular Risk Factors, and Cardiovascular Disease: The West of Scotland Coronary Prevention Study.**

J of Thrombosis and Haemostasis. 2000;84:553–558.

32. Buga GM, Gold ME, Fukuto JM, Ignarro LJ. **Shear stressinduced release of nitric oxide from endothelial cells grown on beads.**

Hypertension. 1991;17:187–193.

33. Martini J, Carpentier B, Chavez Negrete A, Cabrales P, Tsai AG, Intaglietta M. **Beneficial effects due to increasing blood and plasma viscosity**.

Clin Hemorheol Microcirc. 2006;35:51–57.

34. Kimura A, Okumura K, Mokuno S, Numaguchi Y, Matsui H, Murohara T. **Higher viscosity participates in the regulation of coronary flow via nitric oxide and indomethacin-sensitive contracting factor.**

Can J Physiol Pharmacol. 2004;82:1096–1102.

35. Yalcin O, Ulker P, Yavuzer U, Meiselman HJ, Baskurt OK. **Nitric oxide generation by endothelial cells exposed to shear stress in glass tubes perfused with red blood cell suspensions: role of aggregation.**

Am J Physiol Heart Circ Physiol. 2008;294:2098–2105.

36. Tsai AG, Acero C, Nance PR, Cabrales P, Frangos JA, Buerk DG, Intaglietta M. **Elevated plasma viscosity in extreme hemodilution increases perivascular nitric oxide concentration and microvascular perfusion.**

Am J Physiol Heart Circ Physiol. 2005;288:1730–1739.

37. Salazar Vazquez BY, Wettstein R, Cabrales P, Tsai AG, Intaglietta M. **Microvascular experimental evidence on the relative significance of restoring oxygen carrying capacity vs. blood viscosity in shock resuscitation.**

Biochim Biophys Acta. 2008;1784:1421–1427.